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Prediction of the Evolution of Ceftazidime Resistance in Extended-Spectrum β-Lactamase CTX-M-9

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A random mutagenesis technique was used to predict the evolutionary potential of β-lactamase CTX-M-9 toward the acquisition of improved catalytic activity against ceftazidime. Thirty CTX-M mutants were obtained during three rounds of mutagenesis. These mutants conferred 1- to 128-fold-higher MICs of ceftazidime than the parental enzyme CTX-M-9. The CTX-M mutants contained one to six amino acid substitutions. Mutants harbored the substitutions Asp240Gly and Pro167Ser, which were previously observed in clinical CTX-M enzymes. Additional substitutions, notably Arg164His, Asp179Gly, and Arg276Ser, were observed near the active site. The kinetic constants of the three most active mutants revealed two distinct ways of improving catalytic efficiency against ceftazidime. One enzyme had a 17-fold-higher k_{cat} value than CTX-M-9 against ceftazidime. The other two had 75- to 300-fold-lower K_m values than CTX-M-9 against ceftazidime. The current emergence of CTX-M β-lactamases with improved activity against ceftazidime may therefore be the beginning of an evolutionary process which might subsequently generate a great diversity of CTX-M-type ceftazidimases.

The most prevalent mechanism of resistance against β-lactams in gram-negative bacilli is the production of β-lactamases belonging to structural class A (1). Class A β-lactamases are active-site serine enzymes which cleave the amide bond in the β-lactam ring via an acyl-enzyme intermediate.

Oxyimino cephalosporins, such as ceftazidime or cefotaxime, are highly resistant to this hydrolysis by class A penicillinases such as TEM-1, TEM-2, and SHV-1. However, the extensive use of these β-lactams has resulted in the emergence of extended-spectrum β-lactamases (ESBLs). The first ESBLs were derived from the TEM-1/2 and SHV-1 β-lactamases by critical amino acid substitutions which confer hydrolytic activity against oxyimino cephalosporins. The major substitutions are located in two elements of the binding site: the β3 strand at position 238 and the omega loop at positions 164 and 179 (27).

Non-TEM, non-SHV ESBLs designated CTX-M enzymes were identified in the early 1990s (2, 36). The frequency of CTX-M enzymes has increased sharply worldwide since 1995, and they now form a growing family that comprises more than 40 enzymes (3). Most CTX-M enzymes exhibit a much greater hydrolytic efficiency against cefotaxime than against ceftazidime, unlike TEM- and SHV-type ESBLs. However, seven CTX-M mutants harboring point mutations which improve enzymatic efficiency against ceftazidime have been reported recently, suggesting that CTX-Ms are altering their substrate specificity in response to continued antibiotic selective pressure. Five mutants harbor substitution Asp240Gly (4, 5, 16, 29, 31), and two mutants harbor substitutions Pro167Ser/Thr (33, 38). These substitutions have not been previously observed in natural TEM or SHV ESBLs, suggesting that CTX-M enzymes have a singular evolutionary potential. In this work, a random mutagenesis technique was applied to the CTX-M-9-encoding gene to predict whether other substitutions are involved in this evolution process.

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MATERIALS AND METHODS

Bacterial strains and plasmids. An *Escherichia coli* Δ*ampC* strain was constructed from *E. coli* DH5α (Novagen, Darmstadt, Germany). *E. coli* Δ*ampC* and *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany) were used for the cloning experiments. *E. coli* BW25141 was the recipient of the kanamycin resistance cassette (14).

The pKOBEG (10) and pCP20 (13) plasmids were used for the construction of *E. coli* Δ*ampC*. Plasmid pClRio-7 (4) was the original source of the $bla_{CTX-M-9}$ gene, and plasmid pBK-CMV (Stratagene, Amsterdam, The Netherlands) was used for the cloning experiments during the mutagenesis process. The subcloning experiments and the overexpression of CTX-M-encoding genes were performed with a modified pET9a plasmid (24).

Construction of an *E. coli* DH5α Δ*ampC* isogenic mutant. An isogenic mutant of *E. coli* DH5α in which the *ampC* gene was deleted was constructed by the method of Datsenko and Wanner (14). Briefly, *E. coli* DH5α was transformed with pKOBEG plasmid (10) and cultivated at 30°C in Luria-Bertani (LB) broth supplemented with 25 μg/ml chloramphenicol and 1 mM L-arabinose. When the optical density at 620 nm reached 0.5, the bacterial culture was incubated for 20 min at 42°C and for 10 min at 4°C. *E. coli* DH5α was then washed three times with 10% glycerol.

The Flp recognition target-flanked cassette harboring the kanamycin resistance-encoding gene was generated by PCR from *E. coli* BW25141 with the primers MI*ampC*-1 and MI*ampC*-2 (Table 1), which contained sequences homologous to regions adjacent to the *ampC* gene (Fig. 1A). The PCR products were electroporated in the previously glycerol-washed *E. coli* DH5α. The resulting *E. coli* Δ*ampC*::Kan^r, in which the *ampC* gene was replaced by a kanamycin resistance cassette, was selected at 37°C on LB agar containing 50 μg/ml of kanamycin and verified by PCR. After this primary selection, mutants were maintained at 42°C without antibiotics to eliminate the pKOBEG plasmid. The loss of this helper plasmid was verified by a chloramphenicol sensitivity test. The mutant was then transformed with plasmid pCP20, and the kanamycin resistance cassette was deleted using the Flp recognition target system (14). *E. coli* Δ*ampC*::Kan^r(pCP20) was selected at 30°C on LB agar supplemented with 25 μg/ml chloramphenicol and then maintained at 42°C without antibiotic to cure the helper plasmid pCP20. The loss of this helper plasmid and of the kanamycin resistance cassette was checked by a chloramphenicol and kanamycin susceptibility test, and the loss of *ampC* gene was checked by PCR (Fig. 1).

In vitro mutagenesis. The $bla_{CTX-M-9}$ gene was mutagenized by error-prone PCR with the GeneMorph random mutagenesis kit (Stratagene, Amsterdam,

TABLE 1. Oligonucleotides used

Primer	Oligonucleotide sequence (5′→3′)
MI*ampC*-1	GTAAATCCGGCCCGCCTATGGCGGGCCGTT TTGTATGGAAACCAGACCCTGTAG
MI*ampC*-2	CAGCAAGGAAAAGCGGAGAAAAGGTCCGAA AATTCGGACCCGATGGAATTCATA
ampC-1	GGCTATGCGGACATCGCC
ampC-2	GCCTGCTCAAAACTCAAACC
ampC-3	TCTACGGTCTGGCTGCTATCC
ampC-4	TGTCGCTGCGGCAACGAGAG
k2	CGGTGCCCTGAATGAACTGC
kt	CGGCCACAGTCGATGAATCC
k1	CAGTCATAGCCGAATACCCT
pBK-CMV1′	CTAGTGGATCCAAAGAATTCAAAAAGC
pBK-CMV2′	AATTGGGTACACTTACCTGGTACCC
*Nde*ORFM9-A	GGAATTCCATATGGTGACAAAGAGAGTGC
*Sfi*ORFM9-B	GACTATCGGCCTTTTTGGCCTTACAGCCCT TCGGCGAT

The Netherlands) as recommended by the manufacturer. Briefly, 10 ng of plasmid was amplified by PCR with 200 μM each deoxynucleoside triphosphate, 125 ng primers pBK-CMV1′ and pBK-CMV2′ (Table 1), and 2.5 U Mutazyme DNA polymerase. The primers included restriction sites for enzymes KpnI and EcoRI. The mutagenized amplicon was cleaved with these endonucleases (Roche Applied Science, Meylan, France) and cloned into the corresponding restriction sites of plasmid pBK-CMV downstream of the *lac*Z promoter. The recombinant plasmids were transformed into *E. coli* Δ*ampC*, which was then cultured in 1 ml LB broth at 37°C for 1 h.

One hundred microliters of transformed cells was spread on Mueller-Hinton plates supplemented with 32 μg/ml amoxicillin and 20 μg/ml kanamycin to evaluate the total number of CTX-M-producing strains which were screened during the mutagenesis experiment. In parallel, 900 μl of recombinants was selected for growth on Mueller-Hinton agar with increased concentrations of ceftazidime.

Three rounds of mutagenesis were performed consecutively. About 20,000 β-lactamase-producing clones were screened by using 4 μg/ml ceftazidime during the first round. The mutant candidates for the second and third rounds were selected on the basis of inhibition diameters of ≥21 mm for cefotaxime or ≥14 mm for ceftazidime and a number of amino acid substitutions of ≤3 in order to increase the diversity of the recovered substitutions. During the second round, about 10,000 β-lactamase-producing clones were screened from six candidate mutants on agar containing 8 μg/ml of ceftazidime. During the third round, about 10,000 β-lactamase-producing clones were screened from four candidate mutants on agar containing 16 μg/ml of ceftazidime.

Selection of mutants for sequencing. The resistant phenotype of CTX-M mutant-harboring clones was established for β-lactams and kanamycin by the disk diffusion method. These clones were classified into two resistance phenotypes on the basis of inhibition diameters for cefotaxime and ceftazidime. Phenotype 1 exhibited similar inhibition diameters for ceftazidime and cefotaxime, and phenotype 2 exhibited lower inhibition diameters for ceftazidime than for cefotaxime. In both these phenotypes, the clones can be subgrouped on the basis of the inhibition diameter values for β-lactams. $bla_{CTX\text{-}M}$ genes were sequenced from two mutants in each phenotype group.

The sequences were determined by direct sequencing of PCR products, using the dideoxy chain termination procedure with an Applied Biosystems sequencer (ABI 377) (34). The nucleotide and deduced protein sequences were analyzed using software available at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The ClustalW program (http://infobiogen.fr) was used for alignment of multiple protein sequences (39).

Susceptibility to β-lactams. Antibiotic-containing disks were used for antibiotic susceptibility testing by the disk diffusion assay (Sanofi-Diagnostics Pasteur, Marnes la Coquette, France). MICs were determined by a microdilution method on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes la Coquette, France) with an inoculum of 10^4 CFU per spot. MICs of β-lactam antibiotics were determined alone and combined at a fixed concentration of clavulanic acid (2 μg/ml) or tazobactam (4 μg/ml). Antibiotics were provided as powders by Glaxo SmithKline (amoxicillin, ticarcillin, cefuroxime, ceftazidime, and clavulanic acid), Wyeth Laboratories (piperacillin and tazobactam), Eli Lilly (cephalothin), Roussel-Uclaf (cefotaxime and cefpirome), Bristol-Myers-Squibb (aztreonam and cefepime), and Merck Sharp and Dohme-Chibret (imipenem).

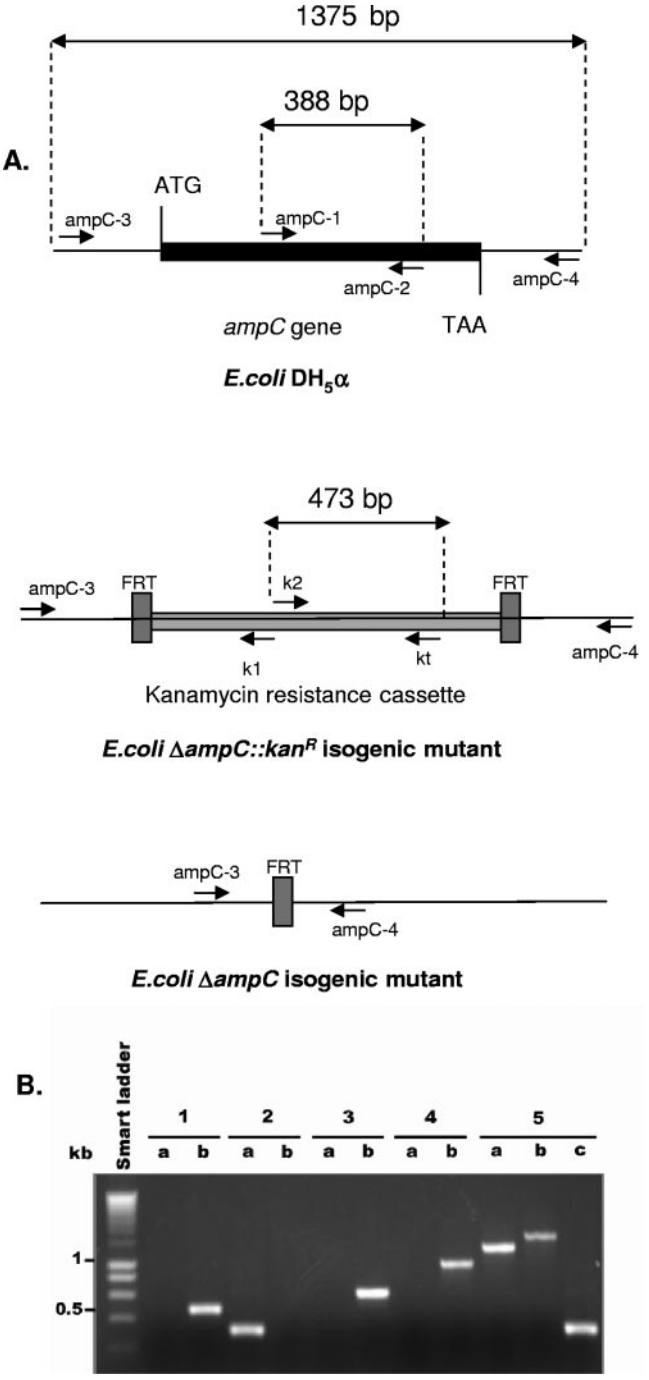

FIG. 1. Deletion of the *ampC* gene from *E. coli* DH5α. (A) Schematic representation of locations of different primers on DNA of *E. coli* DH5α the *E. coli* Δ*ampC*::Kan^r and *E. coli* Δ*ampC* isogenic mutants. (B) PCR amplification product analysis. Amplification products were generated by using specific primers for the kanamycin resistance cassette sequence (k2 and kt) (lanes 1), for the intragenic region of the *ampC* gene (ampC-1 and ampC-2) (lanes 2), for the location of the kanamycin resistance cassette (ampC-3) and k1 [lanes 3] and ampC-4 and k2 [lanes 4], and for the extragenic region of the *ampC* gene (ampC-3 and ampC-4) (lanes 5). (a) *E. coli* DH5α; (b) *E. coli* Δ*ampC*::Kan^r; (c) *E. coli* Δ*ampC*.

TABLE 2. Clones, their phenotypes, and the corresponding enzymes obtained from amino acid substitution in the CTX-M-9 enzyme during three cycles of mutagenesis

Cycle of mutagenesis	Clone	Resistance phenotype	Enzyme	Substitution(s)
First (round A)	A1	1[a]	M-A1	D240G
	A2	1	M-A2	D240G, V29A
	A3	1	M-A3	D240G, I173T
	A4	2[b]	M-A4	L169Q
	A5	2	M-A5	P167S, N106S
	A6	2	M-A6	P167S
	A7	2	M-A7	D179G, N106S, T86A
	A8	2	M-A8	R276S, A231V, R164H
Second (round B)	A1B1	1	M-A1B1	D240G, R276H, Q87L
	A1B2	1	M-A1B1	D240G, A219D
	A4B1	2	M-A4B1	D240G, L169Q
	A5B1	2	M-A5B1	P167S, N106S, T159S
	A6B1	2	M-A6B1	P167S, A109T
	A7B1	2	M-A7B1	D179G, N106S, T86A, A231V, T165I
	A7B2	2	M-A7B2	D179G, N106S, T86A, A231V
	A7B3	2	M-A7B3	D179G, N106S, T86A, R276H
	A8B1	2	M-A8B1	R276S, A231V, R164H, P167S
	A8B2	2	M-A8B2	R276S, A231V, R164H, P167H
	A8B3	2	M-A8B3	R276S, A231V, R164H, G289E, Q188R, D179N
	A8B4	2	M-A8B4	R2768, A231V, R164H, D179Y
Third (round C)	A1B1C1	1	M-A1B1C1	D240G, R276H, Q87L, H112Y, T230I, A231V
	A1B2C1	2	M-A1B2C1	D240G, A219D, G289W, H197R, L169M
	A5B1C1	2	M-A5B1C1	P167S, N106S, T159S, T171S, T209S
	A5B1C2	2	M-A5B1C2	P167S, N106S, T159S, A140V, A231V
	A6B1C1	2	M-A6B1C1	P167S, A109T, G146R, T227A, Q254P
	A6B1C2	2	M-A6B1C2	P167S, A109T, E166V
	A6B1C3	2	M-A6B1C3	P167S, A109T, E201D
	A6B1C4	2	M-A6B1C4	P167S, A109T, A231V
	A6B1C5	2	M-A6B1C5	P167S, A109T, A172V
	A6B1C6	2	M-A6B1C6	P167S, A109T, A77V, P268A

[a] Resistance phenotype 1 exhibits similar MICs for cefotaxime and ceftazidime.
[b] Resistance phenotype 2 exhibits higher MICs for ceftazidime than for cefotaxime.

β-Lactamase preparation. The mutant genes were amplified by PCR from plasmid pBK-CMV with primers NdeORFM9-A and SfiORFM9-B, which included restriction sites for enzymes NdeI and SfiI (Table 1). The PCR products were digested with these two enzymes and were ligated into the corresponding restriction sites of modified plasmid pET9a (24). The resulting plasmids were transformed into *E. coli* BL21(DE3). *E. coli* transformants were selected on Mueller-Hinton agar supplemented with 30 μg kanamycin and 0.5 μg ceftazidime.

CTX-M mutant-encoding genes were overexpressed in *Escherichia coli* BL21(DE3) from pET9 derivative plasmids in 2xYT broth (Qbiogene, Irvine, CA) supplemented with 0.1 mM isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co., St Louis, Mo.), as previously reported (11). After extraction of the enzymes by sonication, the extract was clarified by centrifugation and treatment with DNase I (Roche Applied Science, Meylan, France). CTX-M purification was carried out as previously described (6) by ion-exchange chromatography with an SP Sepharose column (Amersham Pharmacia Biotech) and gel filtration chromatography with a Superose 12 column (Amersham Pharmacia Biotech), using a fast protein liquid chromatography system. The total protein concentration was estimated by the Bio-Rad protein assay (Bio-Rad, Richmond, Calif.), with bovine serum albumin (Sigma Chemical Co., St Louis, Mo.) used as a standard. The purities of enzyme extracts were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6).

Determination of β-lactamase kinetic constants. Steady-state kinetic parameters were determined for mutants exhibiting ceftazidime, cefotaxime, and amoxicillin MICs of ≥64, ≥16, and ≥2048 μg/ml, respectively. The Michaelis constant (K_m) and catalytic activity (k_{cat}) were determined with purified extracts by using a computerized microacidimetric method (23). Bovine serum albumin and Triton X-100 (Sigma Chemical Co., St Louis, Mo.) were added at final concentrations of 50 μg/ml and 0.01%, respectively, to prevent denaturation (20) and aggregation of enzyme M-A6B1C1.

RESULTS

Construction of *E. coli* Δ*ampC*. An *ampC* gene isogenic mutant of *E. coli* DH5α was constructed to eliminate phenotypic variations which may result from AmpC production during in vitro evolution. The *ampC* gene was replaced by kanamycin resistance cassette in *E. coli* Δ*ampC*::Kan^r. The cassette was then eliminated by using the helper plasmid pCP20. Each step of the construction was checked by PCR (Fig. 1). The resulting strain was designated *E. coli* Δ*ampC*.

Collection of mutants. After the first round of mutagenesis, 106 clones were able to grow in the presence of 4 μg/ml ceftazidime. Phenotype 1, which exhibited similar inhibition diameters for ceftazidime and cefotaxime, possessed 81 clones. Phenotype 2, which exhibited a smaller inhibition diameter for ceftazidime than for cefotaxime, possessed 25 clones. The clones belonging to phenotype 2 presented a great diversity of inhibition diameters for the other β-lactams, unlike the clones belonging to phenotype 1, which formed a homogenous group. A second round of mutagenesis was performed from six candidate mutants (one from phenotype 1 and five from phenotype 2), and 123 clones were obtained on agar containing 8 μg/ml ceftazidime; 22 clones belonged to phenotype 1, and 101 clones belonged to phenotype 2. The last round of mutagenesis

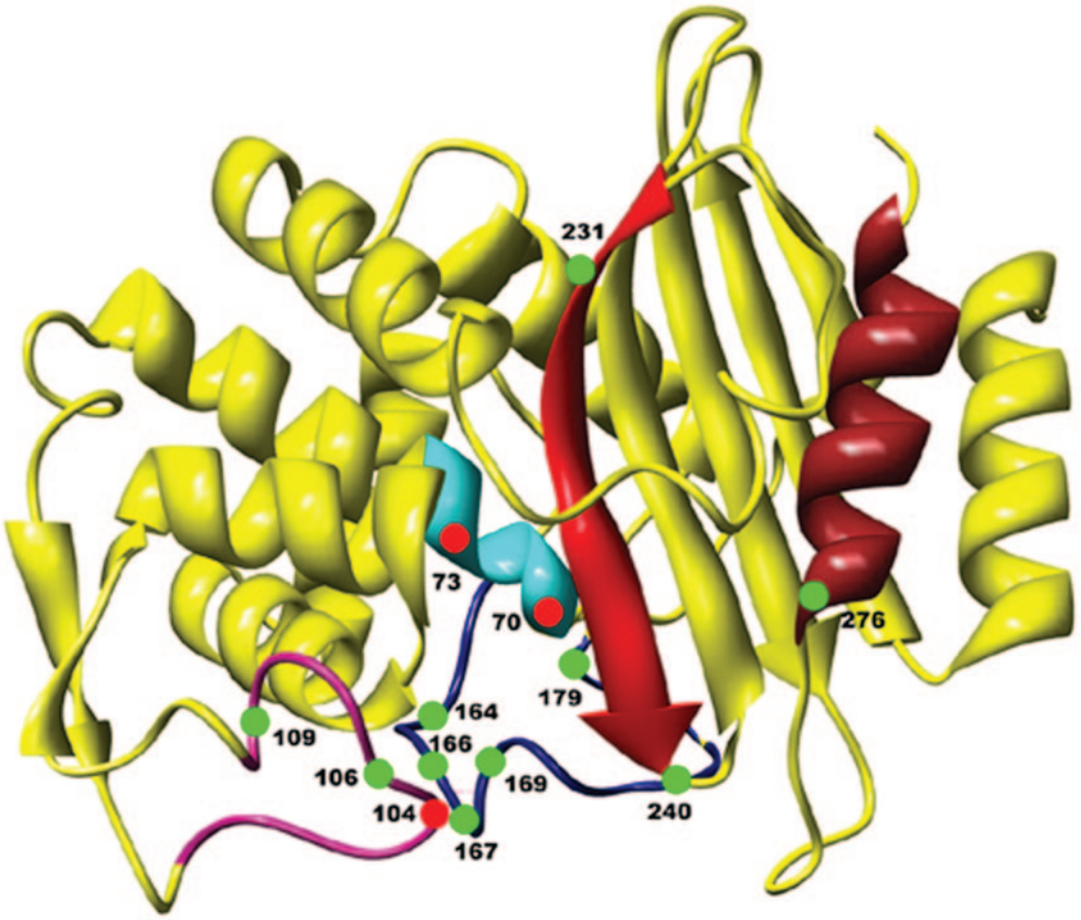

FIG. 2. Crystallographic structure of the CTX-M-9 β-lactamase. The omega loop is in dark blue, the β3 strand is in red, the H11 α-helix is in brown, the N-terminal extremity of the H2 α-helix is in light blue, and the loop between positions 102 and 110 is in purple. The locations of substitutions, which are discussed in the text, are indicated by green circles. Residues Ser70, Lys73, and Asn104 are indicated by red circles. The diagram was drawn with UCSF CHIMERA (30).

was performed from four candidate mutants (two from phenotype 1 and two from phenotype 2); 60 clones had resistance phenotype 1, and 39 others had resistance phenotype 2.

DNA sequencing. Deduced amino acid sequences revealed 38 different substitutions and one to six substitutions per enzyme (Table 2). One to three substitutions per CTX-M mutant were acquired after each cycle of mutagenesis.

All CTX-M-9 mutants associated with resistance phenotype 1 harbored the Asp240Gly substitution, which is located in the β3 strand (Fig. 2). The CTX-M mutants harbored the Asp240Gly substitution alone (M-A1) or associated with substitution Val29Ala (M-A2) or Ile173Thr (M-A3) after the first round of mutagenesis. After the second round, the Asp240Gly substitution was associated with one (Ala219Asp in M-A1B2) or two (Gln87Leu and Arg276His in M-A1B1) additional substitutions. After the last round, three other substitutions appeared in mutants M-A1B2C1 (Gly289Trp, His197Arg, and Leu169Met) and M-A1B1C1 (His112Tyr, Thr230Ile, and Ala231Val).

The mutants involved in resistance phenotype 2 harbored a larger diversity of substitutions than those implicated in resistance phenotype 1. Twenty-four additional substitutions were observed (Table 2). Substitutions at positions 167, 169, 179, and/or 164, which are located in the omega loop, appeared from the first round of mutagenesis (Fig. 2).

The Pro167Ser substitution appeared alone in mutant M-A6 or in association with substitution Asn106Ser in mutant M-A5. Their derivatives M-A5B1 and M-A6B1 harbored the additional substitutions Thr159Ser and Ala109Thr, respectively, and were at the origins of 8 out of 10 mutants obtained during the third round of mutagenesis (M-A5B1C1, M-A5B1C2, and M-A6B1C1 to M-A6B1C6). These mutants harbored a complex combination of substitutions, which comprised substitutions Ala77Val, Gly146Arg, Glu166Val, Thr171Ser, Ala172Val, Glu201Asp, Thr209Ser, Thr227Ala, Ala231Val, Gln254Pro, and Pro268Ala (Table 2).

The Leu169Gln substitution was observed after the first round of mutagenesis alone in mutant M-A4. After the second round of mutagenesis, this substitution was associated with substitution Asp240Gly in its derivative M-A4B1. During the third round of mutagenesis, a second type of substitution, Leu169Met, was observed in mutant M-A1B2C1. This enzyme, which derived from mutant M-A1B2, additionally harbored substitutions Asp240Gly, His197Arg, Ala219Asp, and Gly289Trp.

TABLE 3. β-Lactam MICs for CTX-M-producing mutants of *E. coli* Δ*ampC*

E. coli mutant	MIC (μg/ml) of[a]:													
	AMX	AMC	TIC	TCC	PIP	PIT	CF	CXM	CTX	CAZ	ATM	FEP	CPO	IMP
Δ*ampC*	<256	4	2	2	2	2	4	4	0.06	0.12	0.12	0.06	0.06	0.25
M9[b]	>2,048	16	>2,048	32	256	2	1,024	1,024	16	1	8	2	4	0.25
A1	>2,048	8	>2,048	16	128	2	512	1,024	16	8	16	2	4	0.25
A2	1,024	8	>2,048	16	64	2	256	256	8	8	4	0.5	0.5	0.25
A3	>2,048	2	>2,048	16	256	4	512	1,024	32	16	32	4	8	0.25
A4	256	8	256	8	8	2	16	16	0.5	8	0.5	0.06	0.06	0.25
A5	128	4	64	8	8	2	8	8	0.5	8	0.12	0.12	0.12	0.25
A6	256	8	512	16	8	4	32	16	0.5	8	0.12	0.12	0.12	0.25
A7	256	4	256	8	8	2	8	16	0.25	8	0.5	0.12	0.12	0.25
A8	2,048	16	>2,048	16	128	2	512	256	2	16	1	2	4	0.12
A1B1	>2,048	2	>2,048	16	256	4	256	512	16	8	4	4	8	0.25
A1B2	>2,048	8	>2,048	16	512	2	1,024	2,048	32	16	16	8	16	0.25
A4B1	8	4	32	16	32	4	8	16	8	32	4	1	4	0.25
A5B1	2,048	16	>2,048	16	64	2	64	128	2	16	4	0.5	1	0.25
A6B1	2,048	8	>2,048	16	32	2	128	128	2	32	2	0.5	0.5	0.25
A7B1	512	8	128	8	64	2	32	128	8	64	4	1	1	0.25
A7B2	512	8	512	8	64	2	32	128	8	64	4	1	1	0.25
A7B3	256	8	256	8	16	2	16	64	1	16	1	0.5	1	0.25
A8B1	1,024	8	2,048	32	32	8	128	128	2	64	1	1	2	0.50
A8B2	64	8	256	16	8	4	16	32	2	64	0.5	1	1	0.25
A8B3	2,048	8	>2,048	16	64	2	512	256	16	64	1	2	4	0.25
A8B4	8	8	64	16	8	4	4	16	2	64	0.5	0.5	1	0.25
A1B1C1	>2,048	16	>2,048	32	512	2	1,024	2,048	64	64	16	8	16	0.25
A1B2C1	256	8	512	8	128	2	128	512	16	64	8	4	8	0.25
A5B1C1	1,024	8	1,024	8	16	2	32	128	2	16	2	0.5	0.5	0.25
A5B1C2	>2,048	16	>2,048	16	256	2	1,024	512	8	64	16	2	4	0.25
A6B1C1	>2,048	16	>2,048	64	256	4	1,024	512	16	128	4	2	4	0.25
A6B1C2	8	8	16	16	8	4	8	16	1	32	2	0.5	0.5	0.25
A6B1C3	1,024	8	2,048	16	32	4	128	64	2	16	1	0.25	0.5	0.25
A6B1C4	>2,048	8	>2,048	16	128	4	512	128	8	64	4	0.5	2	0.25
A6B1C5	32	8	64	8	4	2	8	32	2	64	0.5	0.5	0.5	0.25
A6B1C6	2,048	8	>2,048	16	64	2	128	128	8	64	2	1	1	0.25

[a] AMX, amoxicillin; AMC, amoxicillin and clavulanate (2 μg/ml); TIC, ticarcillin; TCC, ticarcillin and clavulanate (2 μg/ml); PIP, piperacillin; PIT, piperacillin and tazobactam (4 μg/ml); CF, cephalothin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; FEP, cefepime; CPO, cefpirome; IMP, imipenem.
[b] M9, CTX-M-9-encoding *E. coli* Δ*ampC*.

The Asp179Gly substitution was associated with substitutions Asn106Ser and Thr86Ala in mutant M-A7. During the second round of mutagenesis, the M-A7-encoding gene was at the origins of the three mutants M-A7B1, M-A7B2, and M-A7B3, which harbored the additional substitutions Thr165Ile, Ala231Val, and/or Arg276His.

The Arg164His substitution was obtained in mutant M-A8 after the first round of mutagenesis in association with substitutions Ala231Val and Arg276Ser. This enzyme was at the origin of four additional mutants (M-A8B1 to M-A8B4) after a second round of mutagenesis. These mutants harbored the substitution at position 164 in combination with other substitutions of the omega loop: Pro167Ser, Pro167His, Asp179Asn, and Asp179Tyr.

MICs of β-lactams. MICs of β-lactams were determined for the 30 clones collected for $bla_{\text{CTX-M}}$ sequencing (Table 3). Overall, ceftazidime MICs increased from 1 μg/ml for CTX-M-9-producing *E. coli* Δ*ampC* to 128 μg/ml for the mutant-producing clones (A6B1C1). MICs of ceftazidime ranged from 8 to 16, 8 to 64, and 16 to 128 μg/ml after the first, second, and third rounds of mutagenesis, respectively. Among the 30 CTX-M-producing clones, 16 had a high level of resistance to ceftazidime (MIC of ≥32 μg/ml). Of these, eight were obtained after the second round of mutagenesis, and the other eight were obtained after the third round. Clone A6B1C1, which had the highest ceftazidime MIC (128 μg/ml), was obtained after three cycles of mutagenesis.

In contrast to CTX-M-9-producing *E. coli* Δ*ampC*, which exhibited higher MICs of cefotaxime than of ceftazidime (16 versus 1 μg/ml), mutant-producing *E. coli* Δ*ampC* clones belonging to resistance phenotype 1 (A1 to A3, A1B1, A1B2, and A1B1C1) exhibited similar MICs of cefotaxime and ceftazidime (8 to 64 versus 8 to 64 μg/ml). MICs of ceftazidime were 8- to 64-fold higher for mutant-producing *E. coli* Δ*ampC* than for the CTX-M-9-producing *E. coli* Δ*ampC*. In comparison with CTX-M-9-producing *E. coli* Δ*ampC*, mutant-producing *E. coli* Δ*ampC* clones of resistance phenotype 1 exhibited no major modification of MICs of penicillins (64 to >2,048 versus 256 to >2,048 μg/ml), cephalothin (256 to 1,024 versus 1,024 μg/ml), cefuroxime (256 to 2,048 versus 1,024 μg/ml), and cefotaxime (8 to 64 versus 16 μg/ml).

The clones belonging to resistance phenotype 2 exhibited higher MICs of ceftazidime than of cefotaxime, unlike CTX-M-9-producing *E. coli* Δ*ampC*. The MICs of cefotaxime for mutant-producing *E. coli* Δ*ampC* defined two groups. Among the clones with cefotaxime MICs of 8 to 16 μg/ml (similar to those of CTX-M-9-producing *E. coli* DH5α-Δ*ampC*), the MICs of amoxicillin were identical to those of CTX-M-9-pro-

TABLE 4. Kinetic parameters of CTX-M-9 and derivative mutants M-A1B1C1, M-A6B1C1, and M-A8B3

Drug	CTX-M-9		M-A1B1C1		M-A6B1C1		M-A8B3	
	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)
Benzylpenicillin	295	25	115	14	12	12	15	4
Amoxicillin	90	20	67	35	5.5	12	7	12
Cephalothin	3,000	150	3,800	140	420	250	200	13
Cefotaxime	450	120	550	200	17	200	2	4[a]
Ceftazidime	2	600[a]	35	450	0.1	8[a]	0.01	2[a]

[a] K_m values were determined as the K_i values by substrate competition with benzylpenicillin.

ducing *E. coli* Δ*ampC* for clones A8B3, A5B1C2, A6B1C1, A6B1C4, and A6B1C6 and were lower (<1,024 μg/ml) for clones A4B1, A1B2C1, A7B1, and A7B2. The others clones exhibited cefotaxime MICs (0.25 to 2 μg/ml) lower than those for CTX-M-9-producing *E. coli* Δ*ampC*. This decrease in cefotaxime MICs was not associated with major modifications of MICs of amoxicillin for clones A8, A5B1, A6B1, A8B1, A5B1C1, and A6B1C3 (1,024 to >2,048 μg/ml), but the MICs of amoxicillin were lower for clones A4, A5, A6, A7, A7B3, A8B2, A8B4, A6B1C2, and A6B1C5 (8 to 256 μg/ml).

For the 30 clones, the MICs of inhibitor-penicillin combinations, aztreonam, cefepime, cefpirome, and imipenem did not increase more than fourfold in comparison with those for CTX-M-9-producing *E. coli* Δ*ampC*.

Kinetic constants. The mutants designated M-A1B1C1, M-A6B1C1, and M-A8B3, were overexpressed in *E. coli* BL21(DE3) from pET9a-derived plasmids and were purified by liquid chromatography. One to 3 milligrams of β-lactamase per liter of culture medium was obtained, and the purity was estimated to be >98%.

The kinetic parameters for these strains are shown in Table 4. M-A1B1C1 exhibited typical enzymatic features of CTX-M mutants. Lower K_m values were obtained for penicillins (K_m, 14 to 35 μM) than for cephalosporins (140 to 450 μM). Cephalothin was the best substrate (k_{cat}, 3,800 s^{-1}), and a higher k_{cat} was observed for cefotaxime (550 s^{-1}) than for ceftazidime (35 s^{-1}). However, the k_{cat} value against ceftazidime was 17-fold higher for M-A1B1C1 than for CTX-M-9. In addition, the ceftazidime K_m value of M-A1B1C1 was lower than that of CTX-M-9 (450 μM versus 600 μM). Conversely, the kinetic constants of M-A6B1C1 and M-A8B3 were different from those of typical CTX-M enzymes, such as CTX-M-9. k_{cat} values against penicillins and cephalothin were 7- to 25-fold lower for the two mutants than for CTX-M-9. k_{cat} values of M-A6B1C1 were also significantly lower than those of CTX-M-9 for oxyimino cephalosporins (cefotaxime, 17 versus 450 s^{-1}; ceftazidime, 0.1 versus 2 s^{-1}). k_{cat} values of M-A8B3 against these substrates were still about 10-fold lower than those of M-A6B1C1. Although the K_m values for cephalothin and cefotaxime were similar for M-A6B1C1 (250 and 200 μM) and CTX-M-9 (150 and 120 μM), the K_m value against ceftazidime was considerably lower for M-A6B1C1 than for CTX-M-9 (8 versus 600 μM). K_m values of M-A8B3 were impressively low for all β-lactams (2 to 13 μM), in particular for oxyimino cephalosporins (2 to 4 μM).

DISCUSSION

CTX-M enzymes exhibited a much greater hydrolytic activity against cefotaxime than against ceftazidime. A random mutagenesis technique was applied to the CTX-M-9-encoding open reading frame to investigate the substitutions which can improve the activity of CTX-M enzymes against ceftazidime. Thirty CTX-M mutants were recovered, and they harbored 38 distinct substitutions at 32 positions. Most positions were located in the vicinity of the binding site and can be categorized into three clusters: positions previously mutated among clinical CTX-M enzymes (positions 167 and 240) (4, 8, 16, 31, 33, 38), mutated positions observed among ceftazidimase TEM/SHV-type ESBLs (positions 164 and 179) (21), and new mutated positions (Fig. 2). These last positions were located close to the entrance of the binding site (positions 106, 109, and 276) and in the omega loop that forms part of the active-site depression (positions 159, 165, 166, 169, 171, 172, and 173).

Substitutions previously observed among clinical CTX-M enzymes. (i) Residue 167. Residue 167 is located in the omega loop. The residue Pro is usually observed at position 167 in class A enzyme. Substitutions at position 167 (Pro167Ser/His) were observed in 14 CTX-M mutants, as in clinical ESBLs CTX-M-19, CTX-M-23, BPS-1m, and OXY-2-5, which exhibited an improved enzymatic activity against ceftazidime (17, 20, 26, 38). All these CTX-M mutants conferred a higher level of resistance to ceftazidime than to cefotaxime. However, this substitution alone was responsible for a dramatic decrease in MICs of penicillins and other cephalosporins. Comparisons of MICs for clones A6, A6B1, and A6B1C4 suggest that substitutions Ala109Thr and Ala231Val may partially compensate for this pernicious action. The effect of substitution Pro167Ser may be due to a decrease in stability (20). Chen et al. showed that substitution Ala231Val caused a 2°C increase in the protein melting temperature of CTX-M-9 and a 1.1-kcal/mol increase in stability (11). This effect may explain the increase in MICs induced by the addition of substitution Ala231Val to substitution Pro167Ser.

(ii) Residue 240. Aspartate 240 is located at the end of the β3 strand, at the entrance of the binding site. The residue at position 240 is not conserved among β-lactamases of class A. Acid residues (Glu or Asp) are observed in TEM-1 and SHV-1 penicillinases and CTX-Ms. Residue Gly240 is present in the ESBLs PER, VEB-1, and BES-1, which have hydrolytic activity against ceftazidime (6, 32). The Asp240Gly substitution was obtained in eight CTX-M mutants, as in natural enzymes CTX-

M-15, CTX-M-16, CTX-M-27, and CTX-M-32, which exhibited improved activity against ceftazidime (4, 8, 16, 31). CTX-M mutant M-A1 has the same amino acid sequence as CTX-M-16. Its derivative M-A1B1C1, which harbored the additional substitutions Ala231Val, Gln87Leu, His112Tyr, Thr230Ile, and Arg276His, conferred high-level resistance against all β-lactams, which therefore is the best compromise in the resistance phenotype. This enzyme had greater hydrolytic activity against ceftazidime (k_{cat}, 35 s^{-1}) than did natural CTX-M enzymes, including those harboring substitutions Pro167Ser and Asp240Gly. These results suggest that the natural CTX-M enzymes harboring the substitution Asp240Gly are the most probable phylum for new mutants conferring the highest level of resistance to β-lactams.

Mutated positions observed among ceftazidimase TEM/SHV-type ESBLs. (i) Residues 164 and 179. Residues Arg164 and Asp179 establish a salt bridge that anchors the base of the omega loop in TEM/SHV penicillinases and CTX-M enzymes (19, 22, 35). Substitutions Arg164Ser/His and Asp179Ala/Gly/Asn/Glu are common among TEM/SHV-type ESBLs (15, 21). These changes are involved in the extension of substrate specificity to extended-spectrum cephalosporins such as ceftazidime by increasing flexibility in the omega loop. However, these substitutions have never been observed in natural CTX-M enzymes. Substitutions at positions 164 and 179 were obtained after one round of mutagenesis. During the three round of mutagenesis, the substitutions were obtained in nine mutants and always in combination with additional substitutions. The substitutions at positions 231 and/or 276 were harbored in eight of these mutants, which conferred a higher level of resistance to ceftazidime than to cefotaxime. Surprisingly, substitutions Arg164His and Asp179Asn/Tyr were both obtained simultaneously in the same mutants. Mutant M-A8B3, which harbored substitutions Arg164His and Asp179Asn in combination with Gln188Arg, Ala231Val, Arg276Ser, and Gly289Glu, conferred 64-fold higher resistance to ceftazidime than the parental enzyme CTX-M-9. However, its hydrolytic activities against oxyimino cephalosporins, including ceftazidime, were greatly inferior to those of CTX-M-9. In fact, the increase in activity against ceftazidime for M-A8B3 resulted in an impressively low K_m value (2 μM, versus 600 μM for CTX-M-9).

(ii) Residue 169. Residue 169 is highly conserved in β-lactamases. In vitro mutagenesis experiments with the TEM-1 enzyme and the recent characterization of the ESBL SHV-57 showed that substitutions Leu169Pro and Leu169Arg resulted in activity against ceftazidime with a concomitantly significant compromise in the resistance to penicillins (25, 40). Two different substitutions were obtained at position 169 in our CTX-M mutants, Leu169Gln and Leu169Met, which seem to confer similar behavior against penicillins and ceftazidime. These substitutions induced an inversion of the resistance phenotype against ceftazidime and cefotaxime in comparison to the phenotype induced by the parental enzyme CTX-M-9, as observed with the other mutants harboring substitutions at positions 164, 167, and 179 located in the omega loop. The association of substitution Asp240Gly with Leu169Gln in mutant A4B1 raised the MICs of ceftazidime and cefotaxime in comparison with those for mutant A4 (ceftazidime MICs, 32 versus 8 μg/ml; cefotaxime MICs, 8 versus 0.5 μg/ml).

Substitutions not observed among clinical CTX-M, TEM, and SHV enzymes. (i) Residue 276. Residue 276 is in the terminal α-11 helix. CTX-M enzymes do not contain Arg244, which interacts with the carboxylate function of substrates and inhibitors in most ESBLs. This interaction is critical for the catalysis and the inhibition process of TEM-1 penicillinase (9). Residue Arg276 of CTX-M enzymes was predicted to be a substitute for Arg244. A substitution at position 276 was obtained during three distinct mutational events. However, the Arg276His substitution in association with substitutions Asp179Gly, Asn106Ser, and Thr86Ala caused only a modest increase in MICs against ceftazidime (8 μg/ml conferred by mutants M-A7 versus 16 μg/ml conferred by mutant M-A7B3). The MICs of inhibitor β-lactam combinations were not significantly modified by these substitutions, in contrast with the substitution at position 244 in TEM enzymes (9).

(ii) Residues 106 and 109. Residues 106 and 109 are located in the loop harboring residue 104, in the vicinity of residue 132, which are implicated in the binding of β-lactams in CTX-M catalytic cavity (35). Residues Asn106 and Ala109, observed in clinical CTX-M enzymes, were replaced in 15 CTX-M mutants by residues Ser106 and Thr109, which are observed in TEM and SHV enzymes (19, 22, 35). Residues 106 and 109 are implicated in the placing of side chains of residues 104 and 132, respectively (35). In association with the substitution Pro167Ser, Ala109Thr increased the ceftazidime MIC (32 μg/ml conferred by mutant M-A6B1 versus 8 μg/ml conferred by mutant M-A6). In addition, the Ala109Thr substitution seems to compensate for the pernicious effect of substitution Pro167Ser on activity against penicillins (32 to >2,048 μg/ml versus 8 to 512 μg/ml) and the other cephalosporins (0.5 to 128 μg/ml versus 0.12 to 32 μg/ml). Mutant M-A6B1C1, which harbored the association Pro167Ser and Ala109Thr in combination with substitutions Gly146Arg, Thr227Ala, and Gln254Pro, had the highest MIC of ceftazidime (128 μg/ml). This was due to the very low K_m (8 μM) of the enzyme with regard to ceftazidime, as observed with mutant M-A8B3.

(iii) Residue 166. The role of Glu166 in both the acylation and deacylation steps of TEM β-lactamases has been well argued from kinetic and modeling studies (27, 28). Recent crystallographic data indicate that the Lys73 side chain may replace Glu166 in the acylation step of CTX-M β-lactamases (11, 12, 18, 37). In this case, a substitution at position 166 would inactivate the CTX-M enzyme. The Glu166Val-harboring mutant M-A6B1C2 conferred a dramatic decrease in the MICs of β-lactams, except for ceftazidime (32 μg/ml), as observed with the Glu166Ala-harboring mutant of PER-1 (7). The unexpected activity of M-A6B1C2 against ceftazidime suggests the intervention of other residues in the process of deacylation of ceftazidime in this mutant, which harbored the additional substitutions Pro167Ser and Ala109Thr. Recent crystallographic data showed that residue Lys73, which is able to interact with residue Ser70 and the catalytic water molecule, was greatly mobile in the enzyme CTX-M-9 (11). Hydrolysis might be catalyzed in M-A6B1C2 by a symmetric process mediated by residue Lys73, which may activate residue Ser70 during the acylation step and the catalytic water molecule during the deacylation step. However, this process seems to be substrate dependent and efficient only against ceftazidime.

In conclusion, 30 clones with ceftazidime MICs (8 to 128

μg/ml) higher than those induced by the CTX-M-9 enzyme (1 μg/ml) were obtained. The substitutions involved in this in vitro evolution include both those previously observed in natural CTX-M enzymes and new substitutions. This wide diversity emphasizes the evolutionary potential of CTX-M enzymes. The substitutions may emerge in the future under selection pressure driven by the therapeutic use of ceftazidime.

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